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Coenzyme Models

40. Spectral and Reactivity Studies of Roseoflavin Analogs: Correlation between Reactivity and Spectral Parameters[1]

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Roseoflavin analogs with a morpholino group or a monoaza-15-crown-5 group at the 8-position [3-methyl-8-morpholino-10-ethylisoalloxazine or 3-methyl-8-(1′,4′,7′,10′-tetraoxa-13′-azacyclopentadec-13′-yl)-10-ethylisoalloxazine (NCrFl), respectively] were synthesized. We have found that the absorption spectra of these roseoflavin analogs are very sensitive to solvent effects: that is, they imparted a red color to polar solvents characteristic of the intramolecular charge transfer from the 8-amino group to the pteridine moiety while they imparted a yellow color to nonpolar solvents characteristic of the inhibition of the charge transfer. The fluorescence spectra were also sensitive to solvent effects: the emission maximum of NCrFl, for example, shifted from 564 nm (water) to 509 nm (benzene) and the fluorescence intensity increased with decreasing solvent polarity. The oxidizing ability, which was estimated by photooxidation of 1-benzyl-1,4-dihydronicotinamide, was well correlated with the absorption maxima: the shorter the maximum wavelength, the more reactive. In particular, the logarithm of the rate constants was linearly correlated with E_T. These results strongly suggest that roseoflavin, inactive in polar media, is possibly activated when it is bound to hydrophobic pockets of enzymes and that the color change can be a quantitative measure of the reactivities.

INTRODUCTION

Roseoflavin, isolated from a culture medium of *Streptomyces* strain No. 768 (*1*), has a dimethylamino group at the 8-position instead of the methyl group in conventional flavin coenzymes (*2, 3*). It was shown that roseoflavin and its 8-*N*-alkyl analogs exhibit more or less an inhibitory effect on the growth of gram-positive

[1] Preliminary communication: S. Shinkai, K. Kameoka, N. Honda, K. Ueda, and O. Manabe, *J. Chem. Soc. Chem. Commun.*, 673 (1985).

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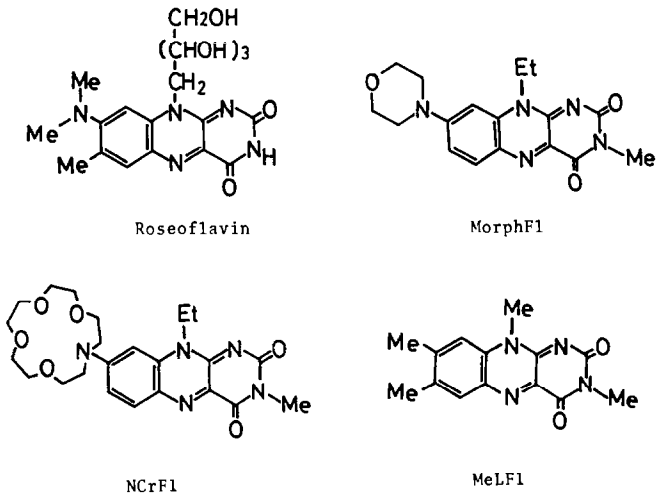


SCHEME 1

bacteria (*4*). This antiriboflavin activity is explained by the fact that the isoalloxazine ring loses its oxidizing ability because of an intramolecular charge transfer from the 8-dimethylamino group to the pteridine moiety (*5*). In fact, roseoflavin and its analogs (Scheme 1) in aqueous solution assume a red color attributable to the charge-transfer band and invariably have redox potentials more negative than those of conventional flavin coenzymes (*4*). Whether roseoflavin shows antiflavin activity under all reaction environments, however, is still a matter of controversy. To obtain an insight into the environmental effects, we are interested in solvent effects on roseoflavin activity. As roseoflavin is not soluble in nonpolar solvents, we synthesized two roseoflavin analogs soluble in most solvents, 3-methyl-8-morpholino-10-ethylisoalloxazine (MorphFl)[3] and 3-methyl-8-(1′,4′,7′,10′-tetraoxa-13′-azacyclopentadec-13′-yl)-10-ethylisoalloxazine (NCrFl), and compared the reactivities with that of 3-methyllumiflavin (MeLFl). We have found that in contrast to the redox properties of conventional flavins, the absorption spectra of these roseoflavin analogs are very sensitive to the solvent effect and the oxidizing ability is well correlated with the shift in the maximum absorption frequencies.

EXPERIMENTAL

Materials. MorphFl and NCrFl were synthesized from 3-methyl-8-chloro-10-ethylisoalloxazine (*6*) by treatment with the corresponding amines. 3-Methyl-8-chloro-10-ethylisoalloxazine (80 mg, 0.28 mmol) was dissolved in morpholine (3.0 g), and the anaerobic mixture was heated at 130°C for 12 h. The resultant red solution was poured into water, and the precipitate (MorphFl) was collected by

[3] Abbreviations used: MorphFl, 3-methyl-8-morpholino-10-ethylisoalloxazine; NCrFl, 3-methyl-8-(1′,4′,7′,10′-tetraoxa-13′-azacyclopentadec-13′-yl)-10-ethylisoalloxazine; MeLFl,3-methyllumiflavin; BNAH, 1-benzyl-1,4-dihydronicotinamide; CrFl, 3,10-dimethyl-1′,4′,7′,10′,13′,16′-hexaoxacyclooctadec-2′-eno[2′,3′-*i*]isoalloxazine; AN, β-anilinonaphthalene; Dabco, diazabicyclo[2.2.2]octane.

suction: mp > 300°C, single spot on TLC, yield 47.3%. Found: C, 59.27; H, 5.64; N, 20.36%. *Anal.* Calcd for $C_{17}H_{19}N_4O_3$: C, 59.80; H, 5.62; N, 20.52%.

3-Methyl-8-chloro-10-ethylisoalloxazine (200 mg, 0.69 mmol) and monoaza-15-crown-5 (333 mg, 0.15 mmol) were dissolved in 8 ml of sulfolane, and the anaerobic reaction mixture was heated at 150°C for 11 h. The progress of the reaction was followed by a TLC method (silica gel–chloroform : methanol (10 : 1, v/v). The dark red solution was mixed with chloroform and washed with water. After concentration *in vacuo*, the reaction mixture was subjected to a TLC separation (silica gel–chloroform : methanol (10 : 1, v/v). Finally, the product (NCrFl) was recrystallized from chloroform–ligroin: mp 215–217°C, one spot on TLC, yield 51.7%. Found: C, 57.96; H, 6.64; N, 14.49%. *Anal.* Calcd for $C_{23}H_{31}N_5O_6$: C, 58.34; H, 6.54; N, 14.79%.

Preparation of 1-benzyl-1,4-dihydronicotinamide (BNAH) was described previously (*7*).

Kinetics. Thermal oxidation of BNAH was carried out under aerobic conditions in water (buffered to pH 8.18 with 30 mM *N*-ethylmorpholine) : methanol (3 : 7, v/v) at 30 ± 0.1°C. The progress of the reaction was followed spectrophotometrically by monitoring the disappearance of the absorption band of BNAH (357 nm). In all cases, the reaction rate was first order in BNAH and flavins.

Photooxidation of BNAH was carried out under anaerobic (N_2) conditions using a Thunberg cuvette. The cuvette containing flavin and BNAH was immersed in a thermostated water bath (30 ± 0.1°C) and irradiated by a 17-W fluorescent lamp. The distance between the cuvette and the lamp was maintained at 14 cm. Progress of the reaction was followed spectrophotometrically by monitoring the reduction of flavin (see Table 2). It is known that light-mediated reactions, which usually consist of several successive steps, do not necessarily obey the first-order equation. In the present system, we confirmed that the disappearance of the absorption bands of the flavins satisfies the first-order equation for up to two half-lives. We thus evaluated the apparent reactivity of the flavins by using the pseudo-first-order rate constants (k_1'). The product analysis using HPLC established that BNAH is quantitatively oxidized to 1-benzylnicotinamide salt.

RESULTS AND DISCUSSION

Phototitration of NCrFl and MorphFl

Kasai *et al.* (*3*) determined spectrophotometrically the pK_a of roseoflavin to be 10.6 and 1.6: the higher pK_a corresponds to the dissociation of 3-NH while the lower pK_a is attributed the protonation of either N(5) or N(8α). They also found that the absorption spectrum of roseoflavin further changes at pH < 0.1, indicating the formation of diprotonated species. We carried out phototitration of NCrFl and MorphFl at 30°C at pH 0.02–6.99. The absorption maxima [498 nm (ε_{max} 46,500) for NCrFl and 492 nm (ε_{max} 36,800) for MorphFl] decreased gradually with decreasing pH of the medium, and new absorption maxima (504 nm for NCrFl and 505 nm for MorphFl) appeared (Fig. 1). The spectra held several tight isosbestic points, indicating that only one equilibrium exists in this pH region. By analysis of

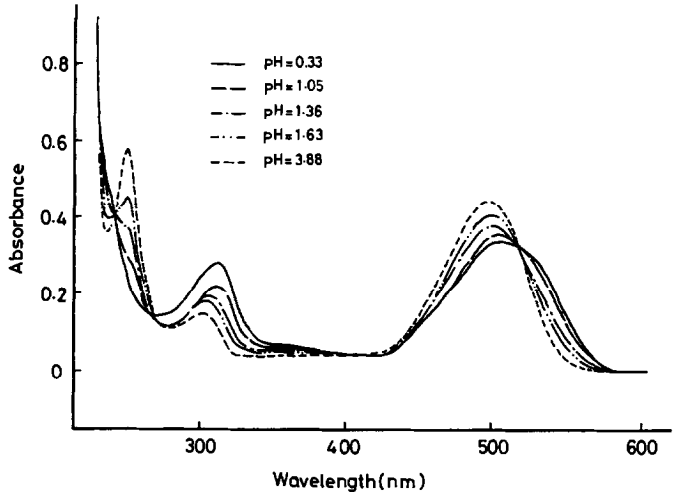


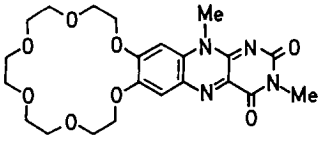
FIG. 1. pH dependence of the absorption spectra of NCrFl (9.95×10^{-6} M) at 30°C in an aqueous system.

the titration curves we obtained pK_a of 1.36 ± 0.02 for NCrFl and 1.20 ± 0.03 for MorphFl. Therefore, one need not take any pH-dependent dissociation of NCrFl and MorphFl into account in the neutral pH region.

Thermal Oxidation in Aqueous Solution

Previously, we reported the first example of a crown ether mimic of flavin coenzymes, 3,10-dimethyl-1′,4′,7′,10′,13′,16′-hexaoxacyclooctadec-2′-eno[2′,3′-*i*]isoalloxazine (CrFl) Scheme 2 (*8, 9*). It was found that in the oxidation of BNAH, the rate constant for CrFl is smaller by one order of magnitude than that for MeLFl. As the logarithm of the rate constants is linearly correlated with the polarographic half-wave potential ($E_{1/2}$) of flavins (*10*), the finding implies that CrFl has an $E_{1/2}$ more negative than that of MeLFl. Interestingly, the rate constant for MeLFl was scarcely affected by the K^+ concentration, whereas that for CrFl was enhanced significantly in the presence of K^+ ion (*9*). This was explained by the complexation of K^+ with the crown ring which would suppress the possible electron donation from the crown ring to the isoalloxazine.

The second-order rate constants (k_2) for the oxidation of BNAH by four flavins are summarized in Table 1. It is seen from Table 1 that (i) NCrFl and MorphFl are further deactivated because of the strong electron-donating ability of the 8-amino group, (ii) the rate constants for NCrFl are improved in the presence of Na^+ (2.1-



SCHEME 2. CrFl.

TABLE 1

THERMAL OXIDATION OF BNAH BY SEVERAL FLAVINS (30°C)[a]

Flavin	Additive	Concentration (M)	k_2 (M^{-1} s^{-1})
MeLFl	—		0.749
MeLFl	K^+	0.20	0.691
CrFl	—		0.096
CrFl	K^+	0.20	0.202
NCrFl	—		0.035
NCrFl	Na^+	0.20	0.073
NCrFl	K^+	0.20	0.057
NCrFl	Rb^+	0.20	0.039
NCrFl	Cs^+	0.15	0.035
MorphFl	—		0.043
MorphFl	Na^+	0.20	0.047
MorphFl	K^+	0.20	0.045

[a] Water (pH 8.18 with 30 mM *N*-ethylmorpholine and HCl) : MeOH = 3 : 7 (v/v).

fold) and K^+ (1.6-fold) but not in the presence of Rb^+ and Cs^+, and (iii) such a salt effect is not observed for MorphFl. These results indicate that the metal–crown interaction is responsible for the small but perceptible rate enhancement in NCrFl.

Effects of Solvent and Metal Ions on Spectroscopic Properties

Flavins usually have two characteristic absorption maxima in the uv (ca. 330 nm, S2 peak) and visible (ca. 440 nm, S1 peak) regions. It has been established from solvent effects that (i) the absorption maximum of S2 can serve as an indicator of solvent polarity since it shifts to shorter wavelengths in nonpolar solvents, and (ii) the absorption maximum of S1 is scarcely affected by solvent polarity, but the spectral shape changes sensitively from a simple Gaussian-type peak in dipolar solvents to a well-resolved two- to three-band fine structure in nonpolar solvents (*11–15*). The absorption spectra of NCrFl and MorphFl in aqueous solution (Table 2 and Fig. 1) feature the weakened S2 band and the shift of the S1 band to longer wavelengths. Thus, they imparted a characteristic red color to water. This color is attributable to a charge-transfer band from the 8-amino groups to the pteridine moiety (*5*).

As described previously, roseoflavin in aqueous solution (λ_{max} 505 nm) assumes a red color attributable to the charge-transfer band (*3*), but it is not soluble in nonpolar solvents. On the other hand, NCrFl and MorphFl showed adequate solubility in most solvents (except saturated hydrocarbon solvents). We found that the absorption maxima of NCrFl and MorphFl shift to shorter wavelengths with decreasing solvent polarity and they produce a yellow to orange color as well as a strong green fluorescence in nonpolar solvents such as dioxane and benzene (Fig. 2). The hypsochromic shift in nonpolar solvents is rationalized in terms of

TABLE 2

ABSORPTION MAXIMA OF ROSEOFLAVIN ANALOGS IN VARIOUS SOLVENTS (30°C)[a]

Solvent	λ_{max} (ε_{max})	
	NCrFl	MorphFl
Water	498 (46,500)	492 (36,800)
Water : MeOH (7 : 3 v/v)	498 (50,900)	492 (44,500)
MeOH	495 (51,700)	485 (40,300)
MeCN	487 (51,500)	480 (45,600)
Ethyl acetate	485 (55,300)	478 (42,900)
	458 (sh)	455 (sh)
THF	486 (55,200)	478 (41,800)
	455 (sh)	457 (sh)
Dioxane	483 (51,600)	473 (37,600)
	455 (sh)	450 (sh)
Benzene	486 (54,300)	479 (39,000)
	457 (31,300)	452 (30,300)
Cyclohexane : benzene	483 (58,300)	477 (36,700)
(1 : 1 v/v)	455 (32,600)	450 (30,300)

[a] sh denotes the appearance of a "shoulder."

the suppression of the intramolecular charge transfer as illustrated in Eq. [1]. The situation is very similar to the well-known solvent effect on the absorption spectra of indophenol derivatives, the absorption maxima of which also shift to shorter wavelengths in nonpolar media (*16*). It is worth mentioning that the resonance structure of the roseoflavin derivatives is quite equivalent to that of the indophenol derivatives (*17*) (see Eq. [2]).

Among solvent polarity parameters, Dimroth's E_T (*18*) would most resemble the resonance structure in Eq. [1], although diphenyl betain, used in their study, has a polar ground state while roseoflavin analogs have nonpolar ground states

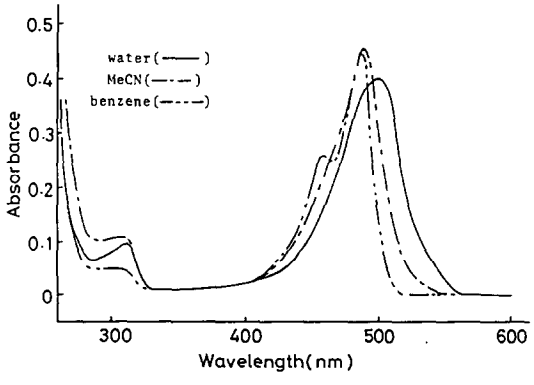
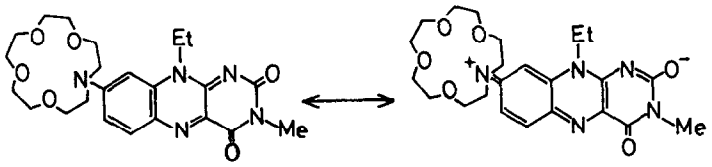
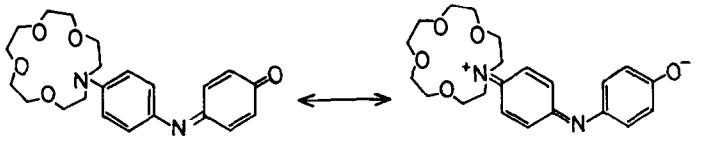


FIG. 2. Absorption spectra of NCrFl (1.15×10^{-5} M) in various solvents at 30°C.



[1]



[2]

and polar excited states. In Fig. 3, the $h\nu$ values calculated from the maximum absorption frequencies of NCrFl and MorphFl are plotted against E_T. It is clearly shown that the $h\nu$ values are correlated almost linearly with Dimroth's E_T.

Here, another method to suppress the intramolecular charge transfer occurred to us: the charge transfer would be offset when the 8-amino groups coordinate to metal ions. In particular, such metal complexes would be greatly stabilized when the 8-amino group is included in a member of a crown ring. In fact, Dix and Vögtle (*17*) found that the absorption maximum of an indophenol derivative bearing a monoaza-15-crown-5 (Eq. [2]) in acetonitrile shifts to shorter wavelengths on the addition of alkali and alkaline earth metal cations. In the present system, the extinction coefficient of the S1 peak of NCrFl was decreased (ca. 2%) on the addition of Na^+ ($[NaClO_4] = 3.51 \times 10^{-3}$ M). The absorption spectrum of MorphFl was not affected by the Na^+ addition. The difference supports the assignment of the spectral change in NCrFl to the Na^+–crown interaction. On the other hand, the addition of alkaline earth metal cations (such as Mg^{2+}, Ca^{2+}, and Ba^{2+}) induced the spectral change not only in NCrFl but also in MorphFl (Fig. 4). The extinction coefficients of the S1 peaks decreased and new broad absorption bands appeared at 500–550 nm. Hence, the spectral change cannot be attributed simply

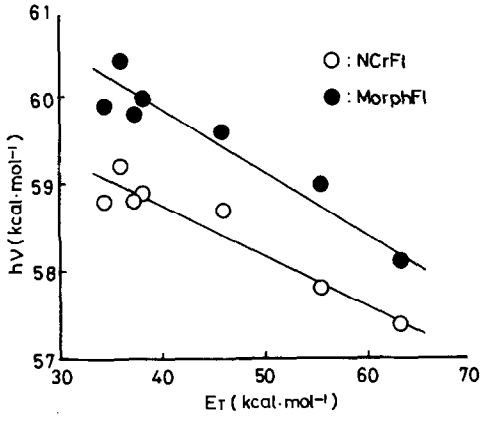


FIG. 3. Plots of Dimroth's E_T vs $h\nu$ calculated from the maximum absorption frequencies of NCrFl and MorphFl.

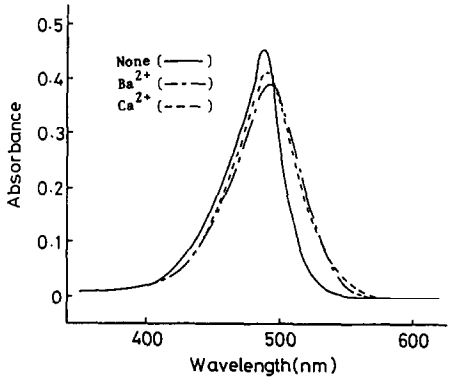


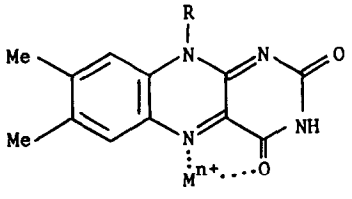
FIG. 4. Absorption spectra of NCrFl (1.15×10^{-5} M) in acetonitrile. $[Ca(SCN)_2] = 7.09 \times 10^{-3}$ M, $[Ba(SCN)_2] = 6.51 \times 10^{-3}$ M.

to the M^{2+}–crown interaction. Although conventional flavins lack the affinity with most metal ions (particularly, in aqueous solution), there are several precedents for the flavin–metal interaction in aprotic solvents (*19–22*). In these precedents, O(4) and N(5) are considered to serve as a metal-chelation site as shown below. Since metal chelation with O(4) induces a shift of the S1 peak to the longer wavelength region (*23–25*), the spectral change in Fig. 4 is not incompatible with the O(4)–N(5) chelation (Scheme 3).

The association constants (K) were estimated from plots of $(1 - (OD/OD_0))/[metal]$ vs OD/OD_0 according to Eq. [3], where ε_c is the molar extinction coefficient of the metal–flavin complex and OD and OD_0 are the optical densities in the presence and the absence of metal ions.

$$\frac{1 - (OD/OD_0)}{[metal]} = K \cdot \frac{OD}{OD_0} - \frac{\varepsilon_c K[flavin]}{OD_0}. \qquad [3]$$

Plots of $(1 - (OD/OD_0))/[metal]$ vs OD/OD_0 gave good straight lines (except a few examples; see Table 3) with a correlation coefficient better than 0.98. We thus estimated the slope (K) from least-squares computation. The results are summarized in Table 3. NCrFl has an association constant of 3×10^2 M^{-1} for Na^+, whereas MorphFl shows no affinity with Na^+. This indicates that the crown ring is the sole chelation site for alkali metal cations. For alkaline earth metal cations, on the other hand, NCrFl can provide two different chelation sites, the crown ring and the O(4)–N(5) site. Careful examination of Table 3 reveals that NCrFl has a



SCHEME 3. O(4)–N(5) metal chelation in conventional flavins.

TABLE 3

ASSOCIATION CONSTANTS (K) IN ACETONITRILE (30°C)

Metal ion	K (M^{-1})		
	NCrFl	MorphFl	BNAH
$NaClO_4$	ca. 3×10^2 [a]	0	0
$Mg(ClO_4)_2$	944	ca. 90 [b]	ca. 10^3 [c]
$Ca(SCN)_2$	251	98	2190
$Ba(SCN)_2$	104	72	253

[a] The spectral change is too small to determine the K accurately.

[b] The plot of $(1 - OD/OD_0)/[metal]$ vs OD/OD_0 (Eq. [3]) did not give a good linear relation.

[c] Cited from A. Ohno, H. Yamamoto, H. Okamoto, T. Osa, S. Oka, and Y. Ohnishi (1977) *Bull. Chem. Soc. Japan* **50**, 2385.

ten times greater association constant for "hard" Mg^{2+} than MorphFl, whereas the difference is only 1.4-fold for relatively "soft" Ba^{2+}. Conceivably, "hard" Mg^{2+} binds mainly to the crown ether moiety, while relatively "soft" Ba^{2+} binds mainly to the O(4)–N(5) site. We previously determined the K for conventional flavins in acetonitrile (*22, 26*): the K values for Mg^{2+} range from 10 to 20 M^{-1}. Since the K values for MorphFl are much greater than these, the metal complexation with roseoflavin analogs is stabilized by the electron-donating effect of the 8-amino group.

The fluorescence properties of NCrFl were found to be exquisitely sensitive to the polarity of the solvent (Table 4 and Fig. 5). In general, the yield of fluorescence increased as the solvent polarity was decreased. For example, the quantum

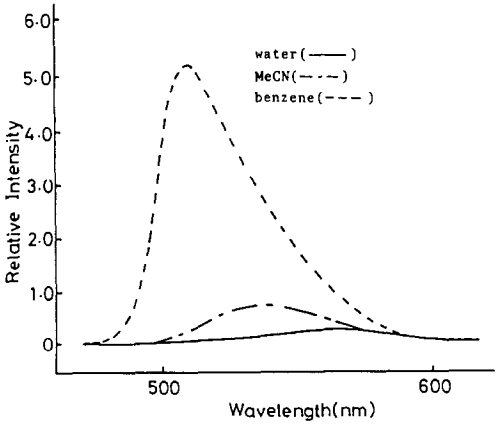


FIG. 5. Fluorescence spectra of NCrFl (2.01×10^{-7} M) in various solvents at 30°C. Excitation 310 nm.

TABLE 4

FLUORESCENCE EMISSION DATA OF NCrFl IN VARIOUS SOLVENT AND EFFECT OF ADDED SALTS ON FLUORESCENCE EMISSION PROPERTIES (30°C)[a]

Solvent	Salt	concn (mM)	λ_{EM} (nm)	Φ_f (%)
Water			565	1.8
MeOH			555	2.1
1-Propanol			548	6.4
MeCN			538	6.1
MeCN	$LiClO_4$	20	558	9.4
MeCN	$NaClO_4$	21	560	6.6
MeCN	$KClO_4$	5	535	6.1
MeCN	$CsClO_4$	5	535	5.7
MeCN	$Mg(ClO_4)_2$	20	557	1.1
MeCN	$Ca(SCN)_2$	18	552	1.3
MeCN[b]	$Ca(SCN)_2$	18	552	0.7
MeCN[b]	$Ba(SCN)_2$	20	567	0.15
t-Butanol			536	19
Chloroform			522	35
Ethyl acetate			510	30
THF			519	36
Dioxane			499	49
Benzene			523	61
Benzene/cyclohexane (1 : 1 v/v)			498	43

[a] Excitation 313 nm. Integrated fluorescence quantum yields were determined relative to fluorescein in 0.1 M NaOH (Φ_f = 0.86).

[b] [2,6-Di-*t*-butyl-4-methylpyridine] = 0.0357 M.

yield of fluorescence emission increased by over 33-fold upon moving from water ($\Phi_f = 0.018$) to benzene ($\Phi_f = 0.61$). The intense fluorescence of NCrFl in benzene nearly rivals that of fluorescein ($\Phi_f = 0.86$) (*37*). The emission maxima of NCrFl also shifted to shorter wavelengths as the solvent polarity was decreased. This trend was complementary to the observed solvent effects on the absorption spectra. In contrast to the striking solvent effects on the fluorescent properties of NCrFl, flavins such as 3-methyllumiflavin undergo only a 2- to 3-fold increase in fluorescence emission as the solvent is varied from water to benzene (*38*). Furthermore, the emission maxima of 3-methyllumiflavin are less sensitive to solvent polarity, shifting from 525 nm in water to 507 nm in benzene.

The interpretation of the results is that the excited singlet state of roseoflavin analogs has considerable dipolar character in polar solvents. In nonpolar solvents the dipolar excited state is destabilized, resulting in hypsochromic shifts in the emission maxima and greatly enhanced fluorescence intensities. Thus in nonpolar solvents, roseoflavin analogs more closely resemble "normal" flavins such as 3-methyllumiflavin in their excited state properties.

To evaluate whether the fluorescence maxima of roseoflavin analogs serve as a measure of solvent polarity, we plotted the ν of NCrFl against ν of β-anilinonaphthalene (AN), a typical fluorescence probe. As shown in Fig. 6, two maximum frequencies showed a good linear relationship (except methanol and water) expressed by

$$\nu_{\text{NCrFl}} = 0.17\ \nu_{\text{AN}} + 1.49 \times 10^{-4}. \quad [4]$$

The finding suggests that roseoflavin can act as a fluorescence probe not only in *in vitro* solvents but also in *in vivo* enzyme active sites.

Solvent Effects on Photooxidation of BNAH

In the foregoing paragraphs, we have demonstrated that spectral properties of NCrFl and MorphFl are very sensitively affected by the solvent effects.

It is interesting to examine the possible correlation between the spectral changes and the reactivities of roseoflavin analogs. We selected the photooxidation of BNAH to evaluate the oxidizing ability of NCrFl and MorphFl (Eq. [5]), for most flavin-dependent thermal reactions are too slow in organic solvents. Usually, photooxidation by flavins proceeds via the triplet state (*27–30*). In the present system, we have confirmed that diazabicylo[2.2.2]octane (Dabco), a well-known quencher for triplet flavins (*30, 31*), does not affect the fluorescence intensity of NCrFl (1.17×10^{-7} M) at [Dabco] = 0.2–1.1 mM but suppresses the photooxidation of BNAH (5.01×10^{-3} M) by NCrFl (1.81×10^{-5} M) in ethyl acetate; the rate constant is apparently smaller by a factor of 3 in the presence of Dabco (1.03×10^{-3} M). The result indicates that this photooxidation also proceeds via the triplet state.

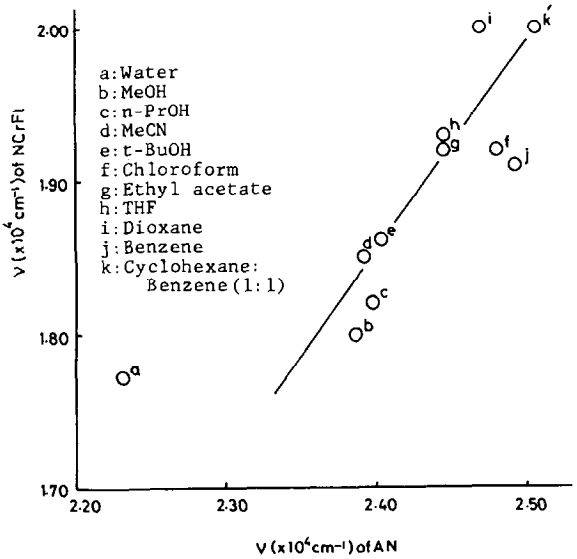
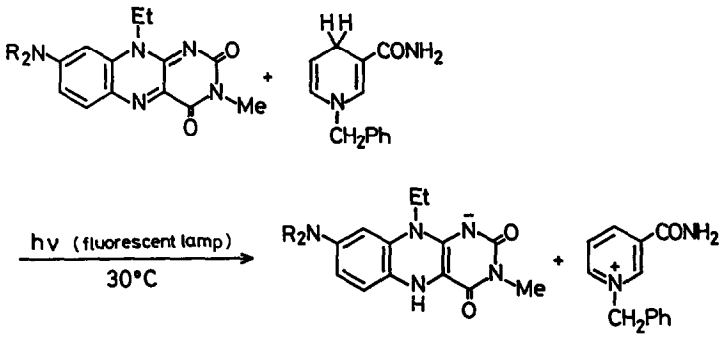


FIG. 6. Plot of fluorescence maximum frequencies of β-anilinonaphthalene (AN) against that of NCrFl.



[5]

The anaerobic photooxidation was followed by spectrophotometric monitoring of the disappearance of the absorption maxima of flavins. Progress of the reaction could be approximated by the first-order rate equation in the presence of excess BNAH, and introduction of O_2 into the final reaction mixture regenerated the oxidized flavins quantitatively. The pseudo-first-order rate constants (k_1') thus obtained are summarized in Table 5.

It is known that flavin-dependent reactions are generally accelerated in polar media, because they have polar transition states without exception. Photooxidation by MeLFl is the case (Table 5). In contrast, photooxidation by NCrFl and MorphFl is very slow in polar solvents such as methanol and acetonitrile while a relatively rapid reaction takes place in nonpolar solvents such as dioxane and benzene. One exceptional case is the aqueous system: the reaction observed in the water–methanol mixed solvent was much faster than that in pure methanol. These results suggest, together with the spectral data, that the oxidizing ability of the roseoflavin analogs in the triplet state is primarily governed by the extent of the intramolecular charge transfer; that is, nonpolar solvents which may suppress

TABLE 5

PSEUDO-FIRST-ORDER RATE CONSTANTS (k_1') FOR THE PHOTOOXIDATION OF BNAH (30°C)[a]

Solvent	$10^3 \cdot k_1'$ (min^{-1})		
	NCrFl	MorphFl	MeLFl
Water : MeOH (3 : 7, v/v)[b]	20	53	
MeOH	ca. 0.1	ca. 0.1	3100
MeCN	0.7	1.0	270
Ethyl acetate	23	30	
THF	60	96	
Dioxane	140	220	320
Benzene	90	170	

[a] [Flavin] = 1.82×10^{-5} M, [BNAH] = 5.00×10^{-3} M, N_2.

[b] Water in the mixed solvent is buffered to pH 8.08 with boric acid (1.0 mM) and NaOH.

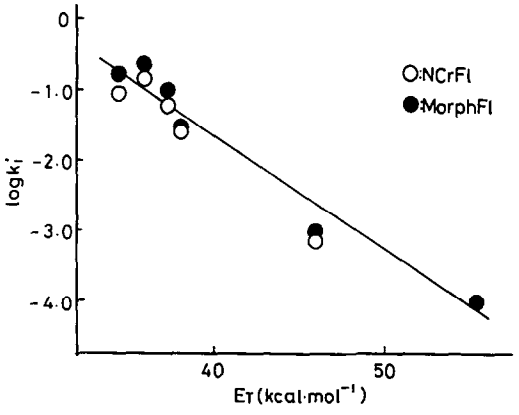


FIG. 7. Plot of Dimroth's E_T vs log k'_i (in min^{-1}).

the charge separation on the isoalloxazine ring can activate the roseoflavin analogs as oxidizing agents. As illustrated in Fig. 7, a plot of log k'_i (in min^{-1}) vs E_T provided a satisfactory linear relationship ($r = 0.97$) expressed by

$$\log k'_i = -0.17E_T + 5.04. \quad [6]$$

The finding indicates that the photooxidizing ability of NCrFl and MorphFl is directly related to their transition energies.

Recently, Kuroda *et al.* (*32*) reported that the photooxidation of alcohols by flavins is accelerated by the addition of $HClO_4$. The finding implies that protonated flavinium ions are more active as oxidation catalysts. Similar activation should be realized by complexation with metal ions. In Table 6, we summarized the k'_i in acetonitrile in the presence of metal ions. The addition of alkali metal cations markedly enhanced the k'_i for NCrFl: Li^+ is the most effective (20- to 32-fold) and Na^+ is next. Since (i) the k'_i for NCrFl increased only to a smaller extent in the presence of Et_4NClO_4 and (ii) the k'_i for MorphFl was unaffected by the addition of Na^+, this rate increase can be ascribed to the metal–crown interaction.

The rate constants were further enhanced in the presence of alkaline earth metal cations: for example, 12- to 68-fold for Ca^{2+} and 45- to 111-fold for Ba^{2+}. Here, one has to pay attention to protic species that might be produced from concomitant water in the reaction medium, because it was very difficult to prepare "anhydrous" $Ca(SCN)_2$ and $Ba(SCN)_2$. To obviate this problem, we added excess 4-methyl-2,6-di-*t*-butylpyridine as proton scavenger. In a separate study, we corroborated that this pyridine has no coordination ability for these metal ions. As a marked rate acceleration is observed even in the presence of the pyridine, this rate enhancement is certainly associated with the flavin–M^{2+} complexation. As recorded in Table 3, spectroscopic studies indicate that these metal ions can interact not only with roseoflavin analogs but also with BNAH. Presumably, these metal ions act as a "bridge," as proposed for Mg^{2+} in the NADH model reduction of carbonyl substrates (*33, 34*), at the photoexcited transition state where a "hy-

TABLE 6

EFFECT OF ADDED METAL SALTS ON THE PHOTOOXIDATION OF BNAH IN ACETONITRILE (30°C)[a]

Additive	Concentration (mM)	$10^3 \cdot k_1^l$ (min^{-1}) NCrFl	MorphFl	MeLFl
None		0.7	1.0	270
$LiClO_4$	4.94	14		
$LiClO_4$	20.6	24		
$NaClO_4$	5.11	11		
$NaClO_4$	21.3	13	1.0	210
$KClO_4$	5.12	4.7		
$CsClO_4$	5.01	8.3		
$Mg(ClO_4)_2$	20.5	3.2	2.9	330
$Ca(SCN)_2$	17.8	47.6[c]	12.4[c]	[b]
$Ba(SCN)_2$	20.1	77.9[c]	44,7[c]	[b]
Et_4NClO_4	20.4	1.7		

[a] [Flavin] = 1.82×10^{-5} M, [BNAH] = 5.00×10^{-3} M, N_2.

[b] The thermal reaction is too fast to estimate the photooxidation rate.

[c] [4-Methyl-2,6-di-*t*-butylpyridine] = 3.57×10^{-2} M.

dride equivalent" is transferred from BNAH to the flavins.[4] The catalytic effect is in the order $Ba^{2+} > Ca^{2+} > Mg^{2+}$. This order is in line with the "softness" of alkaline earth metal cations. The finding suggests, together with the K values in Table 3, that metal ions which preferably bind to the O(4)–N(5) chelation site can catalyze the reaction more effectively.

CONCLUSIONS

The present study can be summarized as follows: roseoflavin analogs, which are inactive in polar media, are activated in nonpolar media because of the suppression of the intramolecular charge transfer from the 8-amino group to the pteridine moiety. This conclusion suggests that roseoflavin, which is believed to be an *inactive coenzyme in flavin clothing*, may possibly be activated when it is bound to hydrophobic pockets of enzymes and that the color change can be a quantitative measure of the reactivities. Therefore, the present system is novel in that the prosthetic group which catalyzes the reaction also serves as a spectroscopic probe exactly at the position where it is bound.

[4] The multistep mechanism involving initial electron transfer is proposed, in some cases, for light-mediated oxidation of BNAH, but the detailed mechanism is still a matter of discussion. See Refs. (*35*, *36*).

REFERENCES

1. OHTANI, S., TAKATSU, M., NAKANO, M., KASAI, S., MIURA, T., AND MATSUI, K. (1974) *J. Antibiot.* **27,** 88.
2. MIURA, R., MATSUI, K., HIROTSU, K., SHIMADA, A., TAKATSU, M., AND OHTANI, S. (1973) *J. Chem. Soc. Chem. Commun.*, 704.
3. KASAI, S., MIURA, R., AND MATSUI, K. (1975) *Bull. Chem. Soc. Japan* **48,** 2877.
4. KASAI, S., KUBO, Y., YAMANAKA, S., HIROTA, T., SATO, H., TSUZUKIDA, Y., AND MATSUI, K. (1978) *J. Nutr. Sci. Vitaminol.* **24,** 339.
5. SONG, P.-S., WALKER, E. B., VIERSTRA, R. D., AND POFF, K. L. (1980) *Photochem. Photobiol.* **32,** 393.
6. YONEDA, F., SHINOZUKA, K., HIROMATSU, K., MATSUSHITA, R., SAKUMA, Y., AND HAMANA, M. (1980) *Chem. Pharm. Bull.* **28,** 3576.
7. SHINKAI, S., ANDO, R., AND KUNITAKE, T. (1975) *Bull Chem. Soc. Japan* **48,** 1914.
8. SHINKAI, S., ISHIKAWA, Y., SHINKAI, H., TSUNO, T., AND MANABE, O. (1983) *Tetrahedron Lett.* **24,** 1539.
9. SHINKAI, S., ISHIKAWA, Y., SHINKAI, H., TSUNO, T., MAKISHIMA, H., UEDA, K., AND MANABE, O. (1984) *J. Amer. Chem. Soc.* **106,** 1801.
10. GASCOIGNE, I. M., AND RADDA, G. K. (1967) *Biochim. Biophys. Acta* **131,** 498.
11. KOZIOL, J. (1966) *Photochem. Photobiol.* **5,** 41.
12. KOZIOL, J. (1969) *Photochem. Photobiol.* **9,** 45.
13. VISSER, A. J. W. G., AND MÜLLER F. (1979) *Helv. Chim. Acta* **62,** 593.
14. KOTAKI, A., NAOI, M., OKUDA, J., AND YAGI, K. (1967) *J. Biochem.* **61,** 404.
15. DUDLEY, K. H., EHRENBERG, A., HEMMERICH, P., AND MÜLLER, F. (1964) *Helv. Chim. Acta* **47,** 1354.
16. BROOKER, L. G. S., AND SPRAGUE, R. H. (1941) *J. Amer. Chem. Soc.* **63,** 3214.
17. DIX, J. P., AND VÖGTLE, F. (1981) *Chem. Ber.* **114,** 639.
18. DIMROTH, K., REICHARDT, C., SIEPMANN, T., AND BOHLMANN, F. (1963) *Justus Liebigs Ann. Chem.* **661,** 1.
19. HEMMERICH, P., MÜLLER, F., AND EHNEVBERG, A. (1965) Oxidases and Related Redox Systems, p. 157, Wiley, New York.
20. FUKUZUMI, S., KURODA, S., AND TANAKA, T. (1984) *Chem. Lett.*, 1375.
21. SHINKAI, S., ISHIKAWA, Y., AND MANABE, O. (1983) *Bull. Chem. Soc. Japan* **56,** 1694.
22. SHINKAI, S., NAKAO, H., UEDA, K., AND MANABE, O. (1984) *Tetrahedron Lett.* **25,** 5295.
23. BAMBERG, P., AND HEMMERICH P. (1961) *Helv. Chim. Acta* **44,** 1001.
24. CLARKE, M. J., DOWLING, J. F., GARAFALO, A. R., AND BRENNAN, T. F. (1979) *J. Amer. Chem. Soc.* **101,** 223 and references cited therein.
25. BAARDA, I. F., AND METZLER, D. E. (1961) *Biochim. Biophys. Acta* **50,** 463.
26. SHINKAI, S., AND NAKAO, H., to be submitted.
27. TRABER, R., WERNER, T., SCHNEIR, S., KRAMER, H. E., KNAPPE, W.-R. AND HEMMERICH, P. (1980) *in* Flavins and Flavoproteins (Yagi, K., and Yamano, T., eds.), p. 431, Japan Scientific Soc. Press, Tokyo.
28. MARUYAMA, K., AND OTSUKI, T. (1983) *Chem. Lett.*, 847.
29. EBERLEIN, G. A., AND POWELL, M. F. (1984) *J. Amer. Chem. Soc.* **106,** 3309.
30. ROKITA, S. E., AND WALSH, C. T. (1984) *J. Amer. Chem. Soc.* **106,** 4589.
31. GOLDBERG, M., PECHT, I., KRAMER, J. E. A., TRABER, R., AND HEMMERICH, P. (1981) *Biochim. Biophys. Acta* **673,** 570.
32. KURODA, S., FUKUZUMI, S., AND TANAKA, T., paper presented at the Annual Meeting of the Chemical Society of Japan, Tokyo, April 1985.
33. OHNISHI, Y., KAGAMI, M., AND OHNO, A. (1975) *J. Amer. Chem. Soc.* **97,** 4766.
34. OHNO, A., KIMURA, T., KIM, S. G., YAMAMOTO, H., AND OKA, S. (1977) *Bioorg. Chem.* **6,** 21.
35. MANRING, L. E., AND PETERS, K. S. (1983) *J. Amer. Chem. Soc.* **105,** 5708.
36. ISHITANI, O., PAC, C., AND SAKURAI, H. (1983) *J. Org. Chem.* **48,** 2941.
37. DAWSON, W. R., AND WINDSOR, M. W. (1968) *J. Phys. Chem.* **72,** 3251.
38. SUN, M., MOORE, T. A., AND SONG, P. S. (1972) *J. Amer. Chem. Soc.* **94,** 1730.